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Functional role of human immunodeficiency virus type 1 *vpu*

(acquired immunodeficiency syndrome/virus replication/regulatory genes)

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ABSTRACT **To investigate the role of *vpu* in the replication and cytopathicity of human immunodeficiency virus type 1 (HIV-1), infectious proviruses were constructed that were isogenic except for the ability to produce the protein product of *vpu*. The *vpu*-encoded protein is shown to decrease the rate of syncytium formation and cell killing in infected CD4$^+$ human T cells, to increase greatly the export of virus particles from infected cells, and to reduce the rate of accumulation of cell-associated viral proteins. The *vpu* protein complements in trans the defect in a *vpu*$^-$ HIV-1 provirus but does not affect the simian immunodeficiency virus, which lacks *vpu*. These observations suggest that *vpu* may contribute to the AIDS epidemic by increasing the transmission efficiency of the virus.**

Human immunodeficiency virus type 1 (HIV-1) encodes at least six proteins in addition to those common to all retroviruses that comprise the virion particle. Two of the supernumerary proteins, the products of the transactivator gene (*tat*) (1–3) and the regulator of expression of virion proteins gene (*rev*) (4–6), are necessary for virus replication. The negative regulatory factor gene (*nef*) (7–10) and the virion infectivity factor gene (*vif*) (11–13) regulate virus expression but are not absolutely required for replication of the virus in CD4$^+$ human T lymphocytes. The functions of the remaining two genes, *vpu* (14–16) and *vpr* (17), have not been clearly defined. Unlike the other genes of HIV-1, no reading frame analogous to *vpu* is found in the genomes of the closely related viruses HIV-2 (18) or simian immunodeficiency virus (SIV) (19, 20). Here we investigate differences in replication of HIV-1 strains constructed to be isogenic except for *vpu*. The results show that *vpu*, like the other supernumerary proteins of HIV-1, regulates the replication and cytopathic effect of the virus.

MATERIALS AND METHODS

Cell Lines and Viruses. The HIV proviral clones HXBc2 and BH10 were provided by R. C. Gallo and F. Wong-Staal at the National Cancer Institute (21, 22). The SIV proviral clone, pFLB10, was isolated by Kyl Myrick at Dana–Farber Cancer Institute (K. Myrick and W.A.H., unpublished). Jurkat and HeLa cells were maintained as described (7, 23).

Site-Directed Mutagenesis. HXBc2 or BH10 segments extending from an *Eco*RI site at position 5329 to the *Xho* I site at position 8480 were cloned into Genescribe vector pTZ19U (24). Single-stranded DNA preparations from these plasmids were used as templates for hybridization of oligonucleotides followed by primer extension and ligation as described (25). Clones were sequenced to confirm the base changes.

DNA Transfections. Jurkat cells (10^7) were treated with 10 μg of plasmid DNA using a DEAE-dextran technique as described (26). Transfections were subsequently given a complete medium change daily.

HeLa cells were seeded at a density of 10^6 cells per 100-mm plate the day before transfection. Cells were transfected with 15 μg of proviral DNA using a modified DEAE-dextran technique as described (27).

HIV p24 and Reverse Transcriptase (RT) Assays. To assay virus-associated p24, 1 ml of culture fluid was collected, passed through a 0.4-μm syringe filter, and centrifuged for 1 hr at 15,000 × *g*. Pelleted virions were resuspended in 10 μl of lysis buffer and assayed by using a commercial HIV p24 radioimmunoassay (RIA) kit (New England Nuclear, no. NEK-040). To assay cell-associated p24, cells were pelleted at 1000 rpm, washed with phosphate-buffered saline (GIBCO), and resuspended in lysis buffer. Typically 2×10^5 Jurkat cells were lysed in 10 μl of buffer, or 10^6 HeLa cells were lysed in 50 μl. The suspensions were centrifuged before assaying to remove insoluble material. Samples for SIV core protein assay were similarly prepared and screened with an antigen capture assay (Coulter Immunology). Samples for assay of supernatant RT activity were collected and assayed as described (28).

Radiolabeling and Immunoprecipitation. Aliquots of infected Jurkat cells were metabolically labeled with [^{35}S]cysteine or [^{35}S]methionine as described (29). Cells were then harvested, lysed, immunoprecipitated with AIDS patient antiserum or anti-*vpu* serum, and electrophoresed through 12.5% SDS/polyacrylamide gels as described (29). Lysate from equivalent numbers of cells was loaded into each lane. Equal volumes of labeling medium from each culture were also collected, filtered, centrifuged to pellet labeled virions, and immunoprecipitated.

Immunofluorescence. HIV-specific immunofluorescence of T cells was carried out as described (30). Numbers of fluorescing cells were counted by eye. Triplicate fields of at least 100 cells were scanned for each sample.

***In Vitro* Transcription and Translation.** SP6 plasmids were linearized at a unique *Eco*RI site in the polylinker 3′ to the HIV insert and used as templates for *in vitro* transcription as described (14, 31). Translation of equimolar amounts of RNA was then performed in rabbit reticulocyte lysate as described (32).

RESULTS

Construction of HIV-1 *vpu* Mutant Proviruses. A set of otherwise isogenic proviruses was constructed that were predicted to differ only in expression of the *vpu*-encoded protein. The infectious HXBc2 provirus, cloned from the IIIB strain of HIV-1, contains an ACG triplet at the 5′ end of *vpu*. No alternate start sites for translation are apparent in the HXBc2 *vpu* frame. By contrast, the BH10 provirus, also derived from IIIB, contains the ATG triplet common to most of the sequenced isolates at the 5′ end of *vpu* and is therefore predicted to produce the *vpu* product (14). For this reason,

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Abbreviations: HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; SIV, simian immunodeficiency virus.

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the segment of HXBc2 located between a unique *Sal* I site at nucleotide 5372 and a *Kpn* I site at nucleotide 5934, a region containing the entire *vpu* reading frame, was replaced with the corresponding segment from BH10 to yield the provirus HXBH10 (Fig. 1). Several differences in nucleotide sequence between HXBc2 and BH10 occur within this segment in addition to the alteration in the *vpu* initiation codon. To produce a pair of proviruses that were truly isogenic except for the *vpu* initiation codon, the ATG of HXBH10 was altered to an ACG by site-directed mutagenesis (24) to yield HXBH10-*vpu*$^-$ (Fig. 1).

Expression of the *vpu* Protein. To determine first whether *vpu* of HXBH10 was capable of expressing a protein *in vitro*, fragments containing *vpu* from HXBc2, HXBH10, and HXBH10-*vpu*$^-$ were each subcloned into a transcription vector 3′ to the SP6 bacteriophage promoter (Fig. 1). RNA transcribed from each plasmid was then used to program reticulocyte translation extracts. RNA transcribed from pEU, an SP6 plasmid containing *vpu* excised from an independently derived HIV-1 clone, ELI (33), was included as a control. As an additional control, the ACG of the HXBc2 *vpu* was corrected to ATG by site-directed mutagenesis to yield HXBc2-*vpu*$^+$. RNA corresponding to the *vpu* region of HXBc2-*vpu*$^+$ was also used in this experiment. Proteins produced were labeled with [^{35}S]methionine and separated by size on SDS/polyacrylamide gels. *vpu* products were detected by using an anti-*vpu* peptide serum previously demonstrated to recognize the *vpu* protein of ELI (14).

As shown in Fig. 2*A*, proteins of apparent molecular mass 20 kDa were precipitated by the anti-*vpu* serum from reaction mixtures containing RNA derived from either the HXBH10 (lane 7) or HXBc2-*vpu*$^+$ (lane 5) *vpu* frame. The peptide used to raise the *vpu* antiserum competed for recognition of both of these 20-kDa proteins (lanes 8 and 6). No proteins were precipitated from reactions programed with RNA derived from *vpu* of either HXBc2 (lanes 3 and 4) or HXBH10-*vpu*$^-$ (lanes 9 and 10). Two previously described proteins of molecular masses 15 and 16 kDa (14) were precipitated from reaction mixtures containing RNA derived from *vpu* of ELI (lanes 1 and 2). The amino acid sequences of the *vpu* proteins of BH10 and ELI are predicted to diverge by about 30% (14), but the proteins are predicted to be of very similar molecular mass. Specifically, the markedly greater polarity of amino acids 56–71 in the BH10 *vpu* protein compared to the corre-

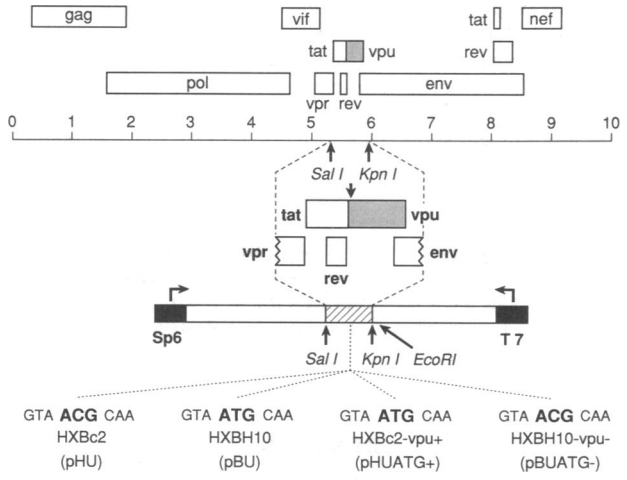

FIG. 1. Plasmid constructs. Segments of the HIV-1 proviruses HXBc2 and BH10 were cloned into an SP6 transcription vector using the *Sal* I and *Kpn* I sites indicated to produce plasmids pHU and pBU, respectively. The alignment of the viral open reading frames within the fragments is shown. Oligonucleotide-directed mutagenesis was carried out upon the HXBc2 and BH10 fragments to remove the *vpu* initiation codon from the BH10 sequence and restore it within the HXBc2 sequence. These mutants were then cloned into the SP6 vector to produce plasmids pHUATG$^+$ and pBUATG$^-$. The DNA sequence at the position of the *vpu* initiation codon (indicated by an arrowhead) within each clone is shown. The mutant HXBc2 as well as both native and mutant BH10 fragments were also cloned into HXBc2 by taking advantage of these same sites to yield the replication-competent proviruses HXBH10, HXBH10-*vpu*$^-$, and HXBc2-*vpu*$^+$. Scale is in kilobase pairs.

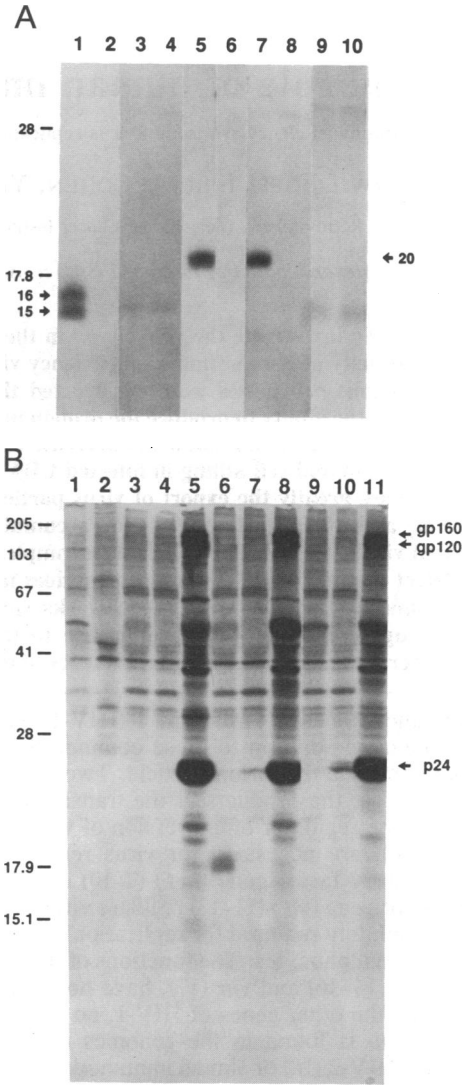

FIG. 2. (*A*) *In vitro* characterization of *vpu* gene products from the different proviruses and proviral mutants. Plasmids pEU (lanes 1 and 2), pHU (lanes 3 and 4), pHUATG$^+$ (lanes 5 and 6), pBU (lanes 7 and 8), and pBUATG$^-$ (lanes 9 and 10) were used as templates for *in vitro* transcription/translation. Labeled products were analyzed by immunoprecipitation with either anti-*vpu* peptide 2 serum (see ref. 14) (lanes 1, 3, 5, 7, and 9) or anti-*vpu* serum in the presence of an excess of peptide 2 (lanes 2, 4, 6, 8, and 10). Immunoprecipitations were analyzed on 12.5% SDS/PAGE. Molecular masses are given in kDa. (*B*) Detection of *vpu* protein in infected Jurkat cultures. Metabolically labeled cells were collected and immunoprecipitated with anti-*vpu* peptide serum. Lanes 1 and 2, control uninfected Jurkat cells immunoprecipitated with anti-*vpu* peptide serum (lane 1) or AIDS patient antiserum (lane 2). Lanes 3–5, HXBc2 infected cells immunoprecipitated with anti-*vpu* peptide serum (lane 3), anti-*vpu* peptide serum following a 1-hr preincubation of the serum with 500 μM of peptide 2 (lane 4), or AIDS patient antiserum (lane 5). Lanes 6–8, HXBH10 infected cells immunoprecipitated with peptide antiserum (lane 6), peptide antiserum plus peptide (lane 7), or AIDS patient antiserum (lane 8). Lanes 9–11, HXBH10-*vpu*$^-$ infected cells immunoprecipitated with peptide antiserum (lane 9), peptide antiserum plus peptide (lane 10), or AIDS patient antiserum (lane 11). Molecular masses are given in kDa.

sponding residues within the ELI protein may reduce the binding of negatively charged SDS molecules sufficiently to account for the decreased electrophoretic mobility of the BH10 product (Temple Smith, personal communication).

These experiments demonstrated that HXBH10 contains an open *vpu* reading frame with an appropriate initiation codon. To determine if a *vpu* product of HXBH10 was produced following virus infection, a CD4$^+$ human T-cell line (Jurkat) previously shown to support HIV-1 replication (7, 11) was transfected with equivalent amounts of HXBH10, HXBH10-*vpu*$^-$, or HXBc2. Six days after transfection, the cells were metabolically labeled with [^{35}S]methionine. The anti-*vpu* serum was then used to screen for *vpu* products in the labeled cell extracts and supernatant viruses. Fig. 2*B* shows that a 20-kDa protein was specifically immunoprecipitated in cell extracts from cultures transfected with HXBH10 but not HXBH10-*vpu*$^-$ or HXBc2. No corresponding band was observed in immunoprecipitates of HXBH10-derived virions (data not shown). This 20-kDa protein has the same electrophoretic mobility as the product synthesized from HXBH10 *vpu* in the reticulocyte lysate. This experiment demonstrates the HXBH10 provirus produces the *vpu* product upon infection of CD4$^+$ T lymphocytes and that HXBc2 does not.

Effect of *vpu* on HIV-1 Cytotoxicity. Parallel cultures of Jurkat cells were transfected with equivalent amounts of HXBH10, HXBH10-*vpu*$^-$, or HXBc2. Cultures were then monitored for total cell number and the appearance of giant multinucleated cells, syncytia. The number of infected cells in the cultures was determined periodically by immunofluorescence using AIDS patient antiserum and fluorescein-conjugated goat anti-human IgG.

A reproducible difference in the effect of virus replication on cell number was observed for *vpu*$^+$ and *vpu*$^-$ viruses (Fig. 3*A*). In all cultures the cell number rose normally until 3 days after transfection. For the next 2 days, the rate of increase in cell number in cultures transfected with either of the *vpu*$^-$ proviruses was markedly lower than that of a mock transfected control culture. By day 6 the number of cells had decreased sharply in cultures infected with *vpu*$^-$ virus. By contrast, the number of cells in the culture transfected with *vpu*$^+$ virus continued to rise normally on days 4 and 5. Not until day 6 did the number of cells in the *vpu*$^+$ culture begin to drop rapidly. Most cells in all three infected cultures were killed by 10 days after transfection. Similar results were obtained in several independent experiments. This difference in cytopathic effect did not appear to be due to a dramatic difference in the rate of spread of the viruses, as the fraction of cells exhibiting HIV-specific fluorescence was similar in all three cultures when measured 3 and 7 days after infection (Fig. 3*A*).

A difference in the kinetics of syncytia formation was also detected in cultures infected with *vpu*$^+$ and *vpu*$^-$ virus. Syncytia were first evident in cultures infected with *vpu*$^-$ virus within 2 days after transfection and reached a maximum by day 3 or 4. Only small numbers of syncytia remained by days 6 and 7 after transfection. In contrast, multinucleated giant cells did not appear in cultures infected with the *vpu*$^+$ virus until 3 days after transfection, and the number of syncytia was maximal between days 5 and 7. No syncytia were evident in any of the cultures by day 9.

Effect of *vpu* on Production of Virus Particles. The amount of virus released into the supernatant of each infected culture was determined. Cells were pelleted and levels of virion-associated RT activity and the major HIV-1 viral capsid protein p24 in the supernatants were determined. In all three cultures, release of virus particles as determined by RT assay was detectable by 3 days after transfection and reached a peak 3–4 days later (Fig. 3*B*). Virus production had decreased sharply in all cultures by day 10. However, at all times after transfection the amount of virus released into the supernatant of cultures infected with the *vpu*$^+$ virus was significantly greater than that in cultures infected with *vpu*$^-$

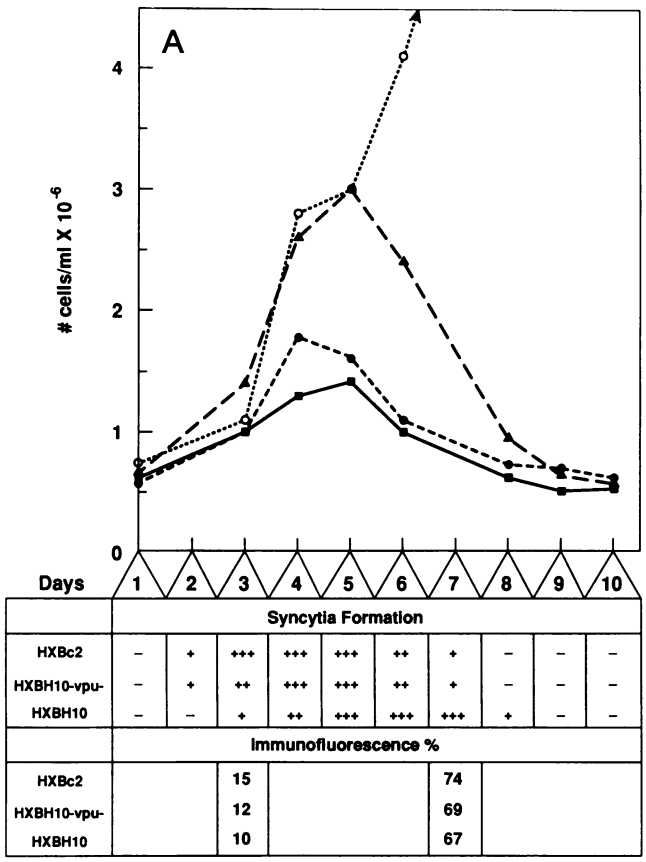

FIG. 3. Replication of *vpu*$^+$ and *vpu*$^-$ viruses within CD4$^+$ lymphocytes. Jurkat cells were transfected on day 0 with either HXBH10, HXBH10-*vpu*$^-$, or HXBc2. Effects of virus infection within each culture were then monitored at regular intervals. (*A*) Cell number, syncytia formation, and HIV-specific immunofluorescence. Cells infected with *vpu*$^+$ virus consistently fluoresced more faintly than cells infected with isogenic *vpu*$^-$ virus. However, the percentage of fluorescing cells in each culture was very similar. (*B*) Supernatant RT activity. ▲, HXBH10; ●, HXBH10-*vpu*$^-$; ■, HXBc2; ○, blank.

virus. At the peak of virus production, there was approximately five times as much virus in the supernatants of cultures infected with *vpu*$^+$ as compared to *vpu*$^-$ virus. Results from p24 RIAs closely paralleled these findings (data not shown). Electron-microscopic examination of infected cells also revealed approximately three times as many budding viruses on the surfaces of cells infected with *vpu*$^+$ as compared to *vpu*$^-$ virus (data not shown).

Ratio of Intracellular to Extracellular Viral Proteins. These observations prompted an investigation of the distribution of viral proteins in the infected cells and supernatants. The ratio of the amount of viral protein associated with the cells as compared to that associated with free virions was measured by using two methods. p24 RIA was performed on equivalent aliquots of detergent-lysed cells to assay levels of cell-associated HIV-1 core protein. Levels of virion-associated p24 in virus pellets centrifuged from the supernatants of these same cultures were also determined. Aliquots of infected cultures were also metabolically labeled with [^{35}S]cysteine on days 2, 4, and 7 after transfection, and the labeled cells and virions were collected the next day and immunoprecipitated with AIDS patient antiserum. The immunoprecipitates were then analyzed on SDS/polyacrylamide gels.

Fig. 4 shows that there was a decrease in the level of all of the viral proteins in immunoprecipitates of cell extracts from cultures infected with *vpu*$^+$ as compared to *vpu*$^-$ virus. The data from the RIAs confirm that there was a decreased amount of HIV-1 core protein present in cells infected with *vpu*$^+$ as compared to *vpu*$^-$ virus (Table 1). The ratio of virion-associated to cell-associated core protein as measured by RIA was consistently 3- to 4-fold higher in cells infected with *vpu*$^+$ as compared to *vpu*$^-$ virus (3.9-fold on day 2, 3.2-fold on day 6, and 3.8-fold on day 10). Densitometry scans of autoradiograms yielded similar results. At every time measured a much greater portion of the total viral protein was

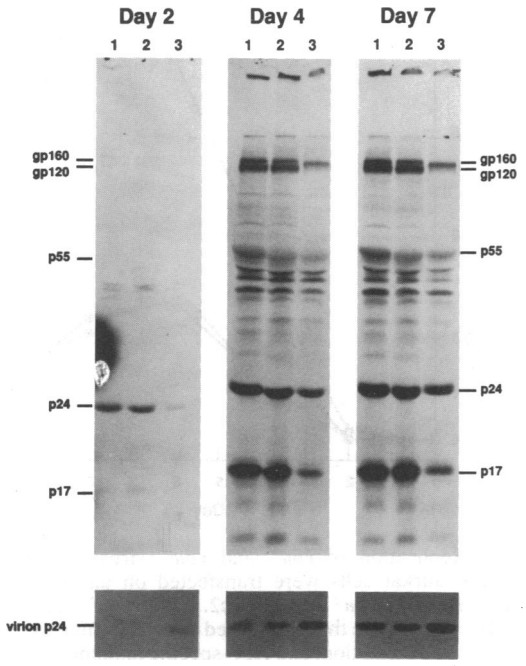

FIG. 4. Immunoprecipitation of serially labeled, infected Jurkat cell lysates with AIDS patient antiserum. Cells from cultures transfected with HXBc2, HXBH10, or HXBH10-*vpu*$^-$ were labeled overnight on days 2, 4, and 7 after transfection with [^{35}S]cysteine, then collected, and immunoprecipitated. Immunoprecipitations of p24 protein from corresponding labeled virus preparations are included at the bottom of each lane. Lanes 1, HXBc2-infected cells. Lanes 2, HXBH10-*vpu*$^-$-infected cells. Lanes 3, HXBH10-infected cells.

Table 1. Jurkat cell-associated p24 (C), virion-associated p24 (V), and their ratios (V/C) at three times after transfection

	p24 core protein levels, ng								
	Day 2			Day 6			Day 10		
Culture	C	V	V/C	C	V	V/C	C	V	V/C
HXBH10-*vpu*$^-$	0.43	0.24	0.56	93	25.0	0.27	87	9.0	0.10
HXBH10	0.19	0.41	2.2	39	34.0	0.87	55	21.5	0.39

C = ng of p24 per 2 × 10^5 cells. V = ng of p24 per viral lysate collected from 1 ml of culture supernatant. A correction has been factored in on days where the HXBH10 culture contained greater numbers of cells than the HXBH10-*vpu*$^-$ culture, so values reflect virion-associated p24 per equivalent numbers of cells in both cases. Values for HXBc2-transfected cells paralleled those for HXBH10-*vpu*$^-$.

present in the supernatant of the culture infected with the *vpu*$^+$ virus as compared to cultures infected with *vpu*$^-$ virus. Similar results were obtained in multiple experiments.

Trans Effect of *vpu*. To study the effect of *vpu* upon partitioning of viral proteins in a system in which the virus can neither spread through the culture nor kill the cells, the *vpu*$^-$ provirus HXBc2 was transfected into either the HeLa-ELI cell line or the HeLa-IIIB cell line (23). The 15-kDa ELI *vpu* product is constitutively expressed in the HeLa-ELI cell line and can be detected by using the anti-*vpu* serum (14). The HeLa-IIIB line does not express a *vpu* product. Both lines produce *tat*, *rev*, and *env* proteins. Neither line contains *gag* or *pol* sequences of HIV-1; therefore, no viruses are produced by these cells in the absence of provirus transfection. Neither line expresses the CD4 antigen; consequently, virions budded from the transfected cells do not infect neighboring cells.

Table 2 shows that the ratio of viral to cell-associated HIV-1 p24 was approximately nine times higher in the HeLa-ELI culture as compared to the HeLa-IIIB cells 48 hr after transfection. A parallel series of experiments conducted using a replication-competent SIV provirus, pFLB10, revealed no significant difference in the partitioning of SIV core protein between cells and virions in the two cell lines 48 hr after transfection (Table 2). Levels of SIV core protein in the cells and culture supernatants were very similar between the two cell lines. This also indicated there was no significant difference in transfection efficiency between the two cell types, in agreement with our previous experience.

DISCUSSION

These experiments demonstrate that the product of *vpu* increases the amount of virus released from infected cells by altering the ratio of intracellular to extracellular viral proteins, effectively enhancing the rate at which HIV-1 proteins are exported from infected cells. The ratio of the intracellular to extracellular viral proteins is altered both in CD4$^+$ human

Table 2. Trans-complementation of *vpu*$^-$ virus in a *vpu*-expressing cell line

	HXBc2			pFLB10		
Cell line	Cells	Virus	Virus/cells	Cells	Virus	Virus/cells
HeLa-IIIB	5	0.05	0.01	0.138	0.028	0.20
HeLa-ELI	4.4	0.38	0.086	0.118	0.035	0.30

HeLa-IIIB and HeLa-ELI cells were transfected with the HIV-1 provirus HXBc2 or the SIV provirus pFLB10. Cells and supernatant virus were collected 48 hr later and assayed for HIV-1 and SIV core proteins. Cell-associated p24 is expressed as ng/10 μl of cell lysate. Virus-associated p24 is expressed as ng per viral lysate pelleted from 1.0 ml of culture fluid. SIV core protein is expressed as absorbance at 450 nm for each sample in an ELISA following subtraction of nonspecific background absorbance (0.10).

T lymphocytes, a natural target cell for HIV-1, and in the epithelial cell line HeLa.

The cytopathic effect of *vpu*$^+$ virus on the CD4$^+$ Jurkat cells is delayed as compared to that observed for *vpu*$^-$ virus. It is possible that the lag in cytopathic effect is linked to the slowed accumulation of cell-associated viral proteins. It has been proposed that a high cellular concentration of a functional envelope glycoprotein, gp120-gp41, is required for both syncytium formation and single cell killing of HIV-1-infected CD4$^+$ T cells. According to this view, the delayed appearance of syncytia and the lag in the decrease in cell number observed in cultures infected with *vpu*$^+$ virus are a result of the decrease in cell-associated gp120-gp41, a consequence of the more rapid export of mature viral proteins. *vpu* therefore enhances virus production primarily by increasing the rate of virus particle export and secondarily by delaying the cytopathic effects of virus infection, permitting infected cells to produce more virus for a longer time.

This study reports the effects of *vpu* on syncytium formation and cell killing and documents the effect of *vpu* upon partitioning of viral proteins between infected cells and supernatant virions. The results are in agreement with the observation of Strebel *et al.* (15) that supernatant RT levels are higher in a CD4$^+$ T-cell line infected with a *vpu*$^+$ as compared to a *vpu*$^-$ virus.

The *vpu* protein is predicted to have a hydrophobic amino terminus (14) and for this reason may be localized to the cell membrane. Yet the *vpu* product does not appear to be present in the mature viral particle. A 1:50 molar ratio of *vpu* protein to p24 protein within virions probably would have been detected in these experiments. A membrane protein may accelerate virus export either by facilitating the aggregation of capsid precursor protein to form a prebudding complex or by accelerating the rate of release of the assembled complex from the cell membrane. The observation that the *vpu* protein is capable of complementing a *vpu*$^-$ HIV-1 virus but not SIV in trans suggests that there is a specific interaction of the *vpu* protein with other HIV-1 viral components.

HIV-1 is the only one of the primate immunodeficiency viruses to contain *vpu*. The presence of *vpu* in HIV-1 may account, at least in part, for the more rapid world-wide distribution of HIV-1 infection as compared to infection with HIV-2. *vpu* may contribute to the world-wide AIDS epidemic by increasing the transmission efficiency of the virus.

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